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Piceatannol attenuates cardiac hypertrophy in an animal model through regulation of the expression and binding of the transcription factor GATA binding factor 6

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ABSTRACT

Piceatannol is found in grapes, passion fruit, and Japanese knotweed. Piceatannol pretreatment suppresses cardiac hypertrophy induced by isoproterenol as assessed by heart weight/body weight ratio, cross-sectional area, and expression of hypertrophic markers. The anti-hypertrophic effect of piceatannol in rat neonatal cardiomyocytes is the same as that in vivo. Piceatannol inhibits lentiviral-GATA6-induced cardiomyocyte hypertrophy. Furthermore, piceatannol reduces the interaction between GATA4 and GATA6 as well as the DNA-binding activity of endogenous GATA6 in the ANP promoter. Our results suggest that piceatannol may be a novel therapeutic agent for the prevention of cardiac hypertrophy.

Structured summary of protein interactions:
GATA6 physically interacts with **GATA4** by anti V5 tag coimmunoprecipitation (View interaction)

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1. Introduction

Stimulated cardiomyocytes may undergo hypertrophy without associated hyperplasia.

In response to the initial stress, cardiomyocytes show a compensatory reaction; under sustained stress, however, they do not reverse to the normal state. Cardiac hypertrophy is characterized by an increase in heart mass, enhanced protein synthesis, highly organized sarcomeres, and reactivation of the fetal gene program [1].

In cardiac hypertrophy, cardiac-specific transcription factors such as GATA4, GATA6, myocyte-specific enhancer factor 2C (MEF2C), homeobox protein Nkx2-5, nuclear factor of activated T-cells (NFAT), serum response factor (SRF), and cAMP-response element-binding protein (CREB) are well known to regulate the cardiac gene program [2]. To date, much evidence has been presented on treating or preventing cardiac hypertrophy through the development of pharmacological targets. A variety of plants and food components have been reported to have beneficial roles in pathological heart diseases. For example, curcumin, quercetin, and resveratrol appear to attenuate myocardial infarction, cardiac hypertrophy [3–5], and heart failure [6].

Piceatannol (trans-3,4,3′,5′-tetrahydroxystilbene), a natural analogue and a metabolite of resveratrol, exists in grapes, red wine, and passion fruit seed [7]. Recently, piceatannol was reported to have pleiotropic roles in inhibiting cancer, inflammation, proliferation, invasion, migration, and adipogenesis [8–10]. Piceatannol is known to be an inhibitor of spleen tyrosine kinase (Syk), phosphoinositide 3-kinase (PI3K), and nuclear factor-κB (NF-κB). Although evidence of a beneficial effect of piceatannol on heart diseases has not been reported, we hypothesized that piceatannol would attenuate cardiac hypertrophy.

Therefore, in the present study, we examined the effects of piceatannol on the development of cardiac hypertrophy in mice and in rat primary cardiomyocytes.

2. Materials and methods

2.1. Reagents and antibodies

Phenylephrine (PE) and isoproterenol (ISP) were purchased from Sigma (Sigma–Aldrich Co., USA). Piceatannol was purchased from Future Chem (FutureChem Co., Korea).

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Anti-Gapdh (sc-32233) and anti-SRF (sc-335) were from Santa Cruz Biotechnology; antibody to atrial natriuretic peptide (anti-ANP) was from Meridian (Meridian Life Science, USA); anti-GATA4 and anti-GATA6 were from Abcam (Abcam, Cambridge Science Park in Cambridge, UK); and anti-sarcomere α-actinin (A7811) was from Sigma.

2.2. Cell cultures

Primary cardiomyocytes from 2-day-old Sprague–Dawley rats were prepared as described previously [11]. Briefly, heart tissue was enzymatically dissociated with collagenase and the cells were isolated using Percoll gradients. Cells were plated on collagen-coated culture dishes.

HEK 293 cells were obtained from the Seoul Korean Cell Line Bank (Seoul, Korea) and were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS).

2.3. Lentiviral GATA6 production and virus infection

Construction of lentiviral GATA6 was prepared as described previously [12]. Briefly, pRRL-RRE-cPPT-CMV-PRE-SIN-mouse GATA6 was prepared by subcloning after PCR amplification of the coding region of GATA6 (restriction enzyme site with *Mlu*I and *Nhe*I from pcDNA3.1-mouse GATA6-V5/His). Lentiviral GFP was used for the control vector and was generated as previously described.

2.4. Luciferase assay

The −2.5 kb GATA6 promoter construct was kindly provided by Prof. Manfred Gessler, University of Würzburg, Würzburg, Germany. Luciferase assays were performed as described previously [13]. pGL3-638 ANP wild, pGL3-638 ANP proximal mutant, pGL3-638 ANP distal mutant, pGL3-3003 ANP promoter, or −2.5 kb GATA6 promoter and pCMV-β-galactosidase were transfected with Lipofectamine Plus reagent (Invitrogen) or PolyFect reagent (Qiagen) according to the manufacturer's instructions. The proximal (−139 to −127) and distal (−297 to −286) mutants of −638 ANP promoter were generated by site-directed mutagenesis as shown in Fig. 4C (upper panel). The cells were incubated in serum-free DMEM for 24 h before treatment and for an additional 24 h with PE or piceatannol. Cell lysates were prepared and luciferase activity was measured with a luciferase assay kit (Promega, USA).

2.5. [^{3}H]Leucine and [^{3}H]thymidine incorporation assay

[^{3}H]Leucine [14] and [^{3}H]thymidine incorporation assays [13] were performed as described previously, respectively.

2.6. Western blot analysis

Western blots were performed as described previously [15]. Cells lysates were prepared with RIPA buffer (150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 50 mM tris–HCl, pH 7.5, 2 mM EDTA, 5 mM NaF) containing protease inhibitors. Proteins were separated by 8–12% SDS-PAGE and were then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were probed with the indicated antibodies and were developed by using Immobilon Western Detection Reagents (Millipore, Billerica, MA, USA).

2.7. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with TriZol reagent (Invitrogen Life Technologies), and 1 μg of RNA was used for the reverse transcription reaction with the Superscript first-strand synthesis system for RT-PCR kit (Invitrogen Life Technologies). Quantification of mRNA amounts was done with the SYBR Green PCR kit (Applied Biosystems, Inc). PCR was performed by using the following oligonucleotide primers:

for *ANP*, sense, 5′-GCTCGAGCAGATCGCAAAAG-3′, and antisense, 5′-GAGTGGGAGAGGTAAGGCCT-3′; for *Gapdh*, sense, 5′-TGCACCAC CAACTGCTTAG-3′, and antisense, 5′-GATGCAGGGATGATGTTC-3′.

2.8. Cell size and sarcomeric organization

Rat neonatal cardiomyocytes were plated on 8-well chambers and fluorescent immunocytochemistry was performed as described previously [14]. After treatment with PE or piceatannol, cells were fixed, permeabilized, and visualized by staining with Texas Red-X phalloidin or sarcomeric α-actinin antibody (1:200) and the cell size was measured by use of NIS Elements Software (Nicon, Japan).

2.9. Chromatin immunoprecipitation (ChIP) and immunoprecipitation (IP)

The ChIP assay was performed by using a kit according to the manufacturer's protocols (Upstate Biotechnology) [16]. Briefly, rat neonatal cardiomyocytes were treated with piceatannol for 6 h. Cells were fixed with 1% formaldehyde for 5–10 min.

The sonicated chromatin was immunoprecipitated with GATA6 (sc-9055x for ChIP) antibody or control goat antibody and then recovered with protein A-agarose/salmon sperm DNA beads. The resulting DNA was amplified by qRT-PCR using primers spanning the GATA binding sites (−504 to −81). Control region was amplified by using primers without potential GATA binding site (exon1, +21 to +178).

For immunoprecipitation, 293T cells were transfected with pcDNA3-GATA4 and pcDNA3-GATA6-V5 by PolyFect reagent (Qiagen). Whole rat cells lysates were incubated with V5 antibody. Immunocomplex was pulled down using Dynabeads Protein G (Invitrogen).

2.10. Animal models and assessment of cross-sectional area in hearts

All protocols were approved by the Animal Care and Use Committee of Chonnam National University. The adult male C57BL/6 mice (6–8 weeks old) used in the current study were purchased from Samtako Bio Korea (Gyeonggi, Korea). Mice were divided into three groups: vehicle-treated control, ISP-injected group, and ISP-injected with piceatannol pretreatment (n = 11–14). Mice were intraperitoneally pretreated with piceatannol (50 mg/kg/day) for 7 days followed by treatment with ISP (10 mg/kg/day) for 3–4 days.

To determine the cross-sectional area of cardiac myocytes, we performed wheat germ agglutinin Alexa Fluor 488 staining (1:200) as described previously [17].

2.11. Statistical analysis

Statistical analysis was performed with either Student's t-test or one-way ANOVA with a Bonferroni post hoc test with software provided by GraphPad Prism version 5.0. Data are presented as means ± S.E.M. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Piceatannol pretreatment inhibits cardiac hypertrophy in isoproterenol-treated mice

Cell cycle progression is implicated in cardiac hypertrophy. Because piceatannol has an anti-proliferative effect, we hypothesized

that piceatannol may prevent cardiac hypertrophy in vivo. We found that treatment with piceatannol and ISP simultaneously for 10 days significantly inhibits heart weight to body weight (HW/BW) ratio (Fig. 1A). In another experiment, pretreatment with piceatannol (50 mg/kg/day) suppressed the increase in the HW/BW ratio (Fig. 1B) induced by isoproterenol (10 mg/kg daily). Rationale for piceatannol dose used in animal experiments is based from several studies showing a suppressive effect of ischemic stroke [18].

As shown in Fig. 1C and D, the cross-sectional area in ISP-stimulated mice pretreated with piceatannol was completely inhibited compared with that in vehicle-treated ISP mice. ISP-induced increases in protein level of ANP were abolished by pretreatment with piceatannol (Fig. 1E and F). Moreover, piceatannol pretreatment inhibited ISP-induced ANP expression in heart sections (Fig. 1G).

3.2. Pretreatment with piceatannol inhibits PE-induced cardiomyocyte hypertrophy

PE, an α-adrenergic agonist, is a well-known inducer of cardiac hypertrophy. Cardiac hypertrophy is characterized by increased

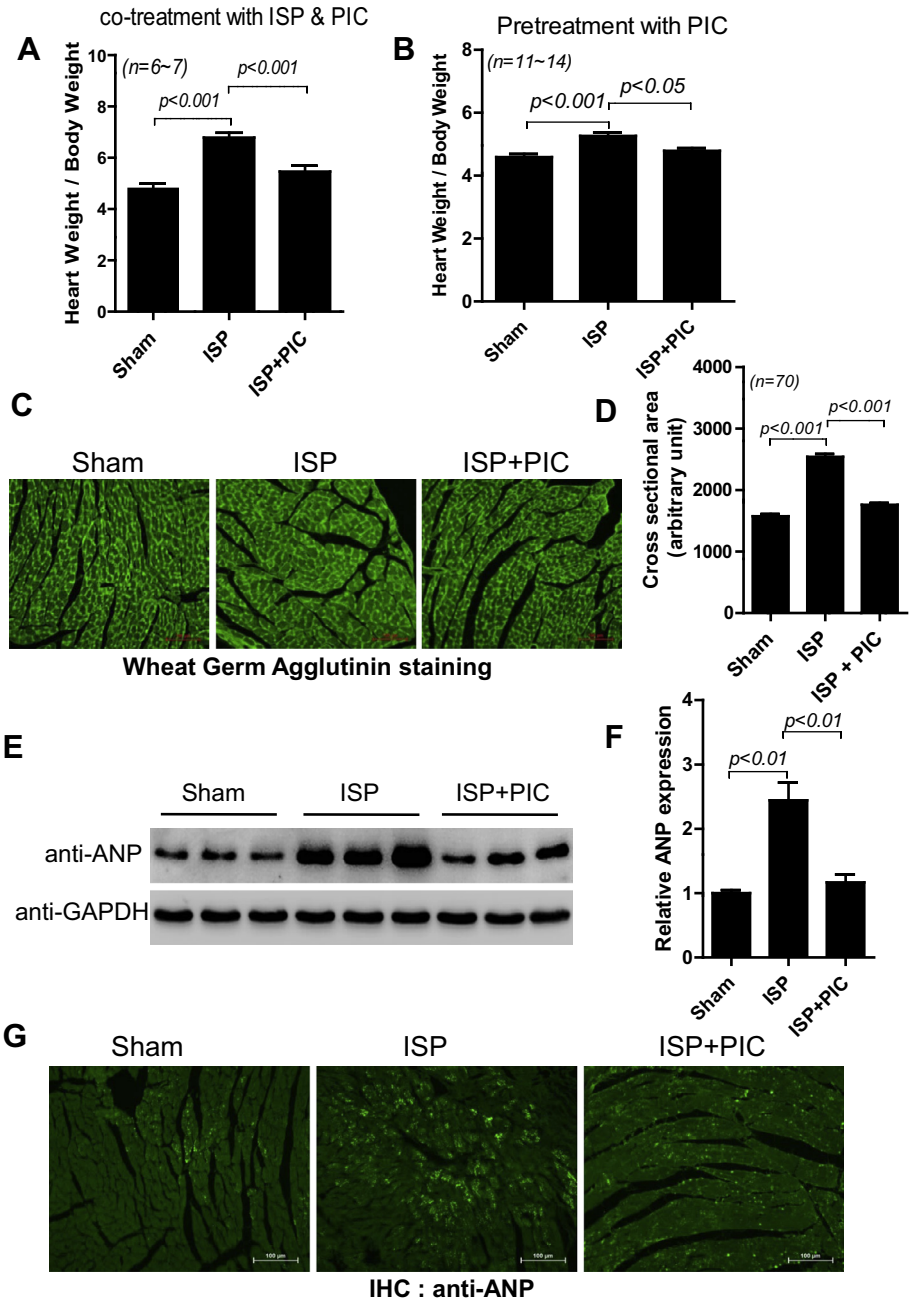

Fig. 1. Piceatannol pretreatment attenuates cardiac hypertrophy in vivo. (A) Mice were intraperitoneally co-treated with piceatannol (50 mg/kg body weight per day) and ISP (10 mg/kg per day) for 10 days. (B) Mice were intraperitoneally pretreated with vehicle or piceatannol at 50 mg/kg body weight daily for 7 consecutive days and then mice were co-administered with piceatannol and isoproterenol for 3–4 days. ISP and PIC indicate isoproternol and piceatannol, respectively. Heart to body weight ratios of the 3 groups. (C) Cardiomyocyte size by wheat germ agglutinin staining in sham mice, ISP mice, and ISP mice treated with piceatannol. (D) Quantitative histomorphometry of 3 groups as measured by cross-sectional areas. (E) Representative Western blot for ANP from heart protein extracts in sham, ISP, and ISP + PIC mice. (F) Relative ANP expression from hearts of the 3 groups. (G) Immunohistochemistry for ANP (green) from hearts. Errors bars in the figure indicate ± S.E.s.

cell size, increased protein synthesis, induction of the fetal gene program, and promoter activity. First, to study the anti-hypertrophic effect of piceatannol in vitro, we measured cell size. As shown in Fig. 2A and B, treatment of cardiomyocytes with PE increased cell size and stress fiber formation, and this increase was significantly inhibited by piceatannol. To further confirm the effect of piceatannol on fetal gene expression, we performed Western blot and qRT-PCR for ANP. We found that piceatannol reduced ANP protein and mRNA expression levels induced by PE (Fig. 2C–E). Furthermore, PE-stimulated [^{3}H]leucine uptake was attenuated by piceatannol (Fig. 2F). To further confirm whether down-regulation of ANP was regulated at the transcript level, we assayed the activity of -3003 ANP promoter. As shown in Fig. 2G, PE increased the activity of the -3003 ANP promoter, and this increase was reduced by treatment with piceatannol.

3.3. Piceatannol inhibits GATA6-mediated cardiac hypertrophy in cardiomyocytes

Next, we wanted to know whether the transcription factors GATA4 and GATA6 mediate cardiac hypertrophy. First, we examined whether GATA4 and GATA6 were induced in response to different stimuli in mice. As shown in Fig. 3A and B, the expression of GATA4 protein was not changed in response to stimulation with PE or ISP. However, GATA6 protein levels were significantly increased at 2 days and then reversed at 3 days after PE injection in mice

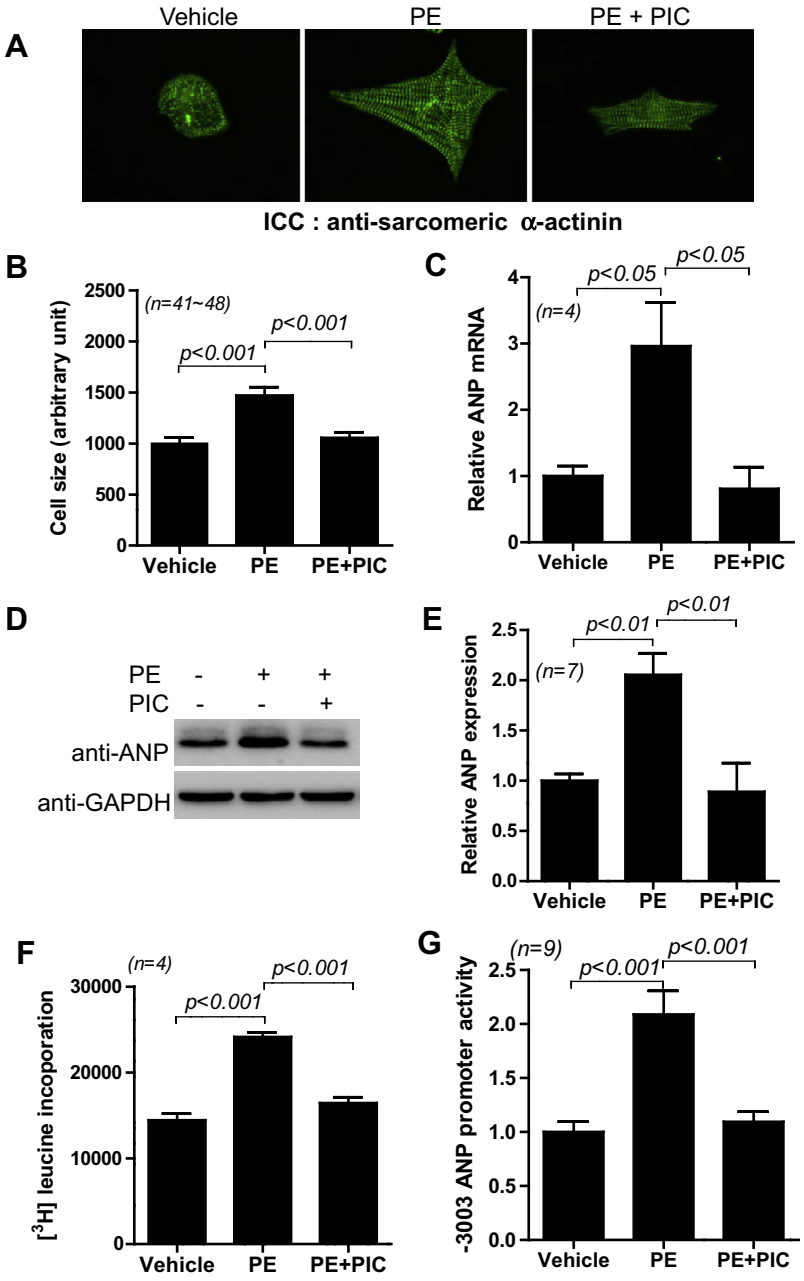

Fig. 2. Piceatannol inhibits cardiomyocyte hypertrophy induced by PE in rat cardiomyocytes. (A) 24 h after cardiomyocytes were treated with piceatannol (PIC) in the presence of phenylephrine (PE), cells were stained with sarcomeric α-actinin. (B) Cell size is indicated by the cell surface areas of the cardiomyocytes by use of NIS Elements software (Nicon, Japan). (C) ANP mRNA levels were measured by qRT-PCR. (D and E) ANP protein expression was determined by Western blot analysis. (F) The protein synthesis rate was evaluated by measuring [^{3}H]leucine incorporation. (G) Rat cardiomyocytes were transfected with -3003 ANP promoter construct with the β-galactosidase as an internal control. Cells were treated with PE or piceatannol. Errors bars in the figure indicate ± S.E.s.

(Fig. 3C). In addition, ISP administration in mice significantly increased the GATA6 protein expression level (Fig. 3D). We also confirmed that GATA6 protein levels were induced within 24 h after PE treatment in cardiomyocytes (data not shown). We found that PE induced GATA6 promoter activity, whereas piceatannol treatment blocked the increased activity (Fig. 3E).

We then generated a lentivirus GATA6 construct to investigate the role of GATA6 in cardiomyocytes. Western blots showed that the expression of GATA6 was induced by lentiviral infection. As shown in Fig. 3F, piceatannol attenuated endogenous GATA6 protein expression as well as exogenously expressed GATA6 in cardiomyocytes. We next asked whether GATA6 could induce

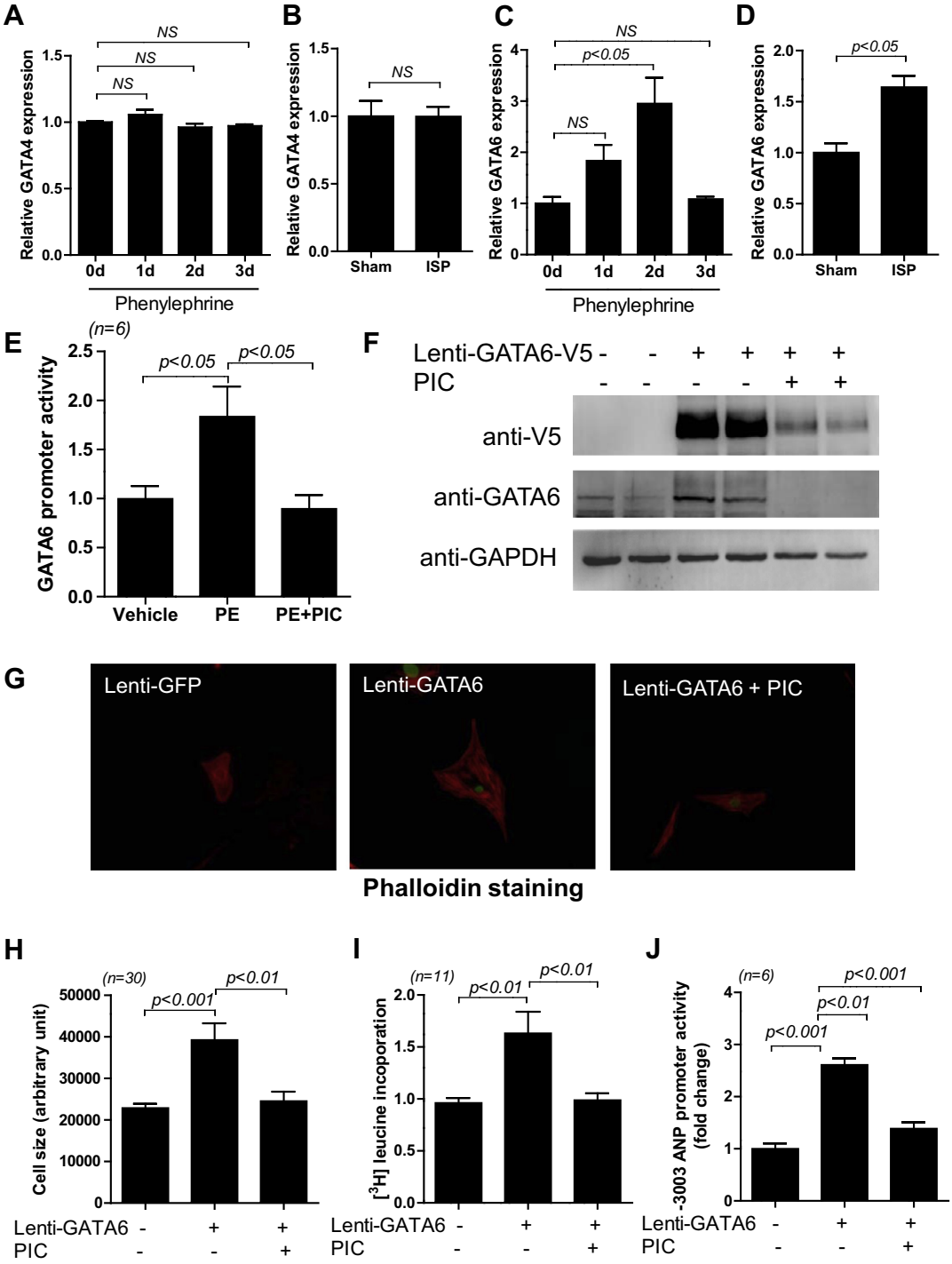

Fig. 3. Piceatannol suppresses GATA6-induced cardiac hypertrophy. (A) Mice were subjected to administration of PE up to 3 days. GATA4 protein was examined. (B) GATA4 protein expression was evaluated at 3 days after injection of ISP in mice. (C) GATA6 protein expression profile was assessed in mice heart with PE treatment. (D) ISP-treated mice showed enhanced expression of GATA6 protein. (E) Rat cardiomyocytes were transfected with GATA6 promoter and β-galactosidase and incubated in serum-free DMEM for 24 h. After treatment with PE or piceatannol for 24 h, luciferase activity was evaluated. (F) Cardiomyocytes were infected with lentiviral GATA6 and then treated with piceatannol. GATA6 expression was evaluated by Western blot. (G and H) After phalloidin staining of cardiomyocytes, cell size was measured by using the NIS Elements Software. (I) The protein synthesis rate was determined by [^{3}H] leucine incorporation. Piceatannol suppressed the increased protein synthesis induced by lentiviral GATA6. (J) -3003 ANP promoter activity was determined after lentiviral GATA6 infection with piceatannol. Errors bars in the figure indicate ± S.E.s.

cardiac hypertrophy. Lentivirus-GATA6 increased cell size in cardiomyocytes, which was completely inhibited by piceatannol treatment (Fig. 3G and H). Furthermore, GATA6 significantly increased [^{3}H]leucine uptake and -3003 ANP promoter activity in cardiomyocytes, and these increases were dramatically reduced by piceatannol treatment (Fig. 3I and J).

3.4. Piceatannol inhibits cardiac hypertrophy via suppression of the binding activity of GATA4 and GATA6

Our results showed that GATA6 was only induced in response to adrenergic agonists such as PE or ISP in cardiomyocytes. However, GATA4 is a well-known mediator of cardiac hypertrophy. We

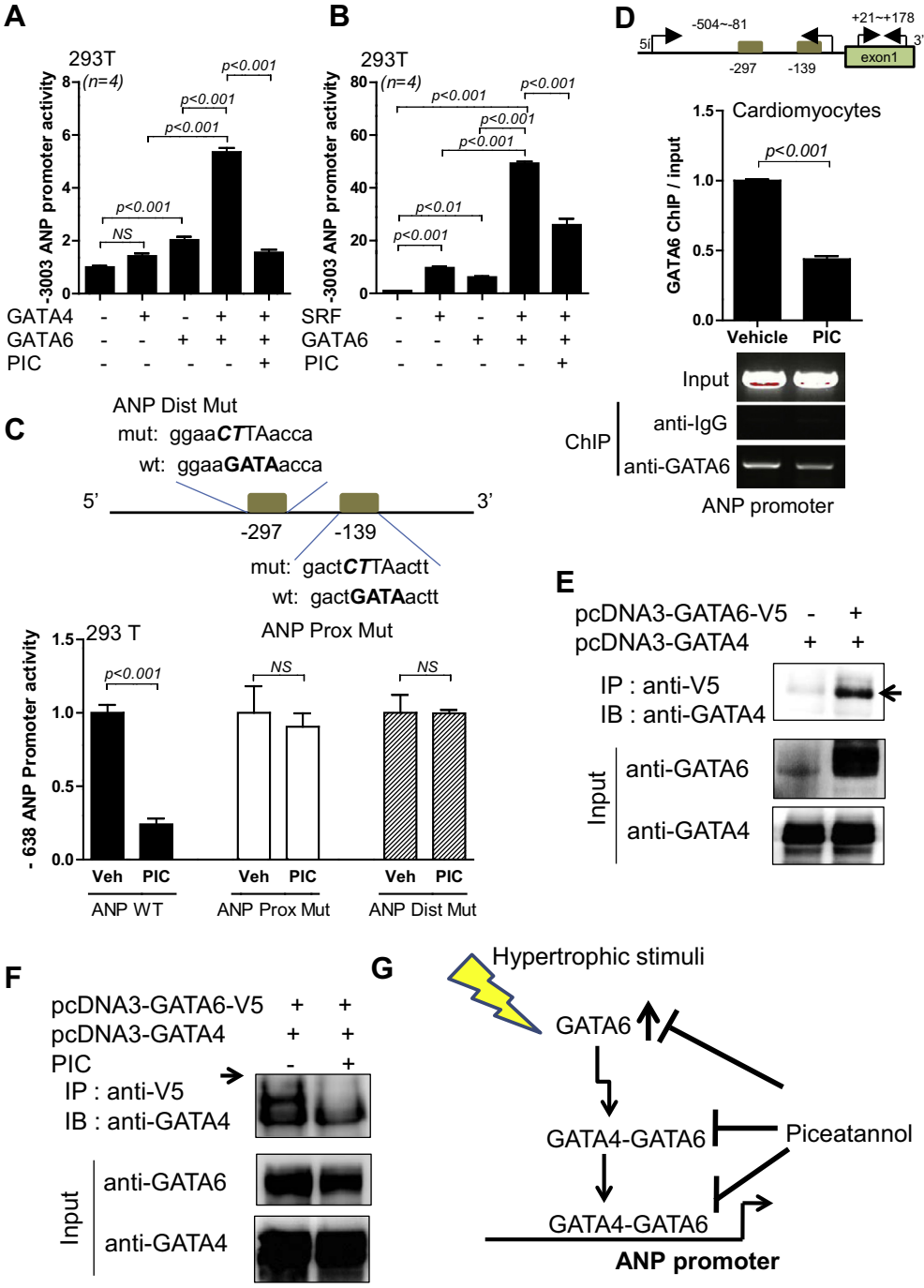

Fig. 4. Piceatannol blocks the interaction and binding activity of GATA4 and GATA6. 293T cells were co-transfected with -3003 ANP promoter construct and pcDNA3-GATA4 or pcDNA3-SRF or pcDNA3-GATA6-V5. At 24 h after transfection, cells were treated with vehicle or piceatannol (5 μM) for 1 day. (A) Piceatannol suppressed the potentiation of ANP promoter mediated by GATA4 and GATA6. (B) Piceatannol attenuated SRF/GATA6-mediated transactivation of ANP promoter. (C) GATA-binding element (−297 or −139 bp) was shown in -638 ANP promoter. The GATA-binding element was mutated by substituting the two nucleotides as shown in the black italic capital letters. After transfection of -638 ANP wild, -638 ANP proximal mutant, and -638 ANP distal mutant promoter construct in 293T cells, the cells were treated with piceatannol and promoter activity was assayed. (D) The binding activity of endogenous GATA6 in the ANP promoter was reduced by piceatannol, as determined by chromatin immunoprecipitation (ChIP). (E) Immunoprecipitation shows the physical interaction of GATA4 and GATA6 in 293 T cells. (F) The interaction of GATA4 with GATA6 was blocked by treatment with piceatannol. (G) Diagram showing the inhibitory action of piceatannol in cardiac hypertrophy. Hypertrophic stimuli induce the expression of GATA6 and then strengthen the interaction of GATA4 and GATA6. This interaction is prevented by piceatannol. Errors bars in the figure indicate ± S.E.s.

hypothesized that GATA6 could synergistically induce cardiac hypertrophy through interaction with other cardiac transcription factors. We demonstrated that GATA6 potentiated the -3003 full ANP promoter activity with co-transfection of GATA4 in 293T cells. However, piceatannol treatment significantly inhibited the GATA4/GATA6-mediated transactivation of the -3003 ANP promoter (Fig. 4A). We also showed that GATA6 potentiated SRF-induced -3003 ANP promoter activity and that the cooperative synergy was attenuated by piceatannol treatment (Fig. 4B). We further determined whether the GATA binding element is necessary in the piceatannol-induced regulation of the ANP promoter. As shown in Fig. 4C, piceatannol inhibited wild-type ANP promoter activity, but failed to inhibit promoter activity when the GATA-binding element was mutated. Next, we examined whether the activity of GATA6 binding to the ANP promoter region was regulated by piceatannol. As shown in Fig. 4D, piceatannol treatment reduced GATA6 binding in the ANP promoter. We further investigated whether the potentiation of GATA4/GATA6-mediated transactivation of the ANP promoter was attributable to binding of GATA4–GATA6. Immunoprecipitation revealed that GATA4 physically interacted with GATA6 in 293T cells (Fig. 4E). Treatment of piceatannol in 293T cells co-transfected with GATA4 and GATA6 reduced the interaction between GATA4 and GATA6 (Fig. 4F).

4. Discussion

In the present study, we demonstrated both in vivo and in vitro that the phytochemical piceatannol inhibits cardiac hypertrophy. Our findings are summarized in Fig. 4G. As shown, hypertrophic stimuli such as an α- or β-adrenergic agonist induce expression of the transcription factor GATA6 in mice and in cardiomyocytes. GATA6 induces cardiomyocyte hypertrophy and this induction of hypertrophy is potentiated by interaction between GATA6 and GATA4. Pretreatment with piceatannol can inhibit the cardiac hypertrophy. Our findings showed that piceatannol decreases GATA6 expression induced by hypertrophic stimuli. In addition, piceatannol inhibits cardiac hypertrophy through a reduction in the binding activity between GATA4 and GATA6.

The transcription factors GATA4, GATA6, SRF, and MEF2 are known to be important in regulating cardiac hypertrophy. We noticed that GATA6 protein levels were increased by hypertrophic stimuli with PE or ISP in vivo and against PE stimuli in vitro. We examined whether GATA6 overexpression by lentiviral GATA6 infection was sufficient for inducing cardiac hypertrophy. We showed that GATA6 increased myocyte cell size and the protein synthesis rate as well as ANP promoter activity in cultured cardiomyocytes. Our findings are consistent with previous data indicating that adenovirus GATA6 infection or GATA6 transgene expression in mice is sufficient to induce a hypertrophic response [19].

One possible mechanism involved in the inhibitory effect of piceatannol on cardiac hypertrophy may be the regulation of the transcription factors GATA4 and GATA6. First, we found that piceatannol decreases the protein levels of GATA6 induced by hypertrophic stimuli in cardiomyocytes or mice. Second, we demonstrated that piceatannol prevents the synergistic effect of GATA4 and GATA6. For example, interestingly, piceatannol inhibited the potentiated transactivation of the ANP promoter in cells co-transfected with GATA4 and GATA6. In addition, by use of a GATA binding mutant luciferase construct, we revealed that the GATA binding region is required for activation of the ANP promoter. Third, we showed that piceatannol can block the physical interaction of GATA4 and GATA6. Consistent with our result, GATA4 was shown to physically interact with GATA6 in postnatal cardiomyocytes [20]. Last, we showed that piceatannol can inhibit the DNA binding activity of the GATA binding element in the ANP promoter.

In conclusion, we have elucidated that piceatannol can prevent cardiac hypertrophy in vivo and in vitro. Indeed, our studies showed that piceatannol inhibits the development of cardiac hypertrophy through regulation of the expression and activity of transcriptional factor GATA6.

Disclosures

None.

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